# Possible Inhibitory Mechanism of Alpha-fetoprotein on PHA-induced Lymphocyte Transformation

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Alpha-fetoprotein (AFP), a fetal serum glycoprotein, is the dominant serum protein in early embryonic life and has been described for every mammalian species studied[1]. The protein reappears in substancial concentration in the adult with hepatocellular carcinoma and other conditions[2], yet very little is known about its biologic function.

Recently, the ability of AFP of mouse or human origin to suppress certain T cell-dependent immune reactions in vitro has been reported[2~5]. These findings on AFP have potentially important implications as to our understanding of the maternal-foetal immunological relationship, the development of immune capabilities in the foetus and new born, and of certain diseases[6~8] where immunological hyporeactivity and elevations in AFP often occur concomitantly.

However, the mechanisms through which AFP exerts its immunoregulatory influence is largely unknown. In vitro lymphocyte transformation by PHA is thought to be closely associated with the non-specific T cell function of immunological mechanism. In addition, it is a particularly suitable model to study mechanism of cell activation[10].

A few indicaters on the interaction of AFP with lymphocyte may be obtained using direct immunofluorescence[11]. Although this approach has been exploited on a number of occasions, some of the results are not satisfactory, because of one or more reasons. For example, the immunofluorescence technique was insensitive, AFP-lymphocyte interaction was studied in the absence of antigen, and the experiments were performed using different conditions.

In the present study, therefore, we extend this concept by demonstrating the interaction of AFP with lymphocyte and PHA under the normal culture conditions using $^{125}I$ labelled AFP. The mode of action of non-specific immunosuppressionis described.

## MATERIALS AND METHODS

### *In Vitro lymphocyte cultures*

A uniform technique for culturing, labelling, and harvesting the cell was used throughout the entire study. Lymphocyte suspensions were prepared from human heparinized peripheral blood by the Ficoll-Isopaque gradient method and were cultured in microtest plates as described by Hartzman *et al*[12]. All cells were washed 3 times and cultured in Eagle's minimal essential medium (MEM) supplemented with glutamine.

Triplicate cultures of responding cells containing $10^5$ lymphocyte/well were set up by use of micropipet in a total volume of 0.1 ml of medium to which 0.01 ml of pooled AB serum and 1.35 $\mu$g of PHA-P (Difco Lab. Detroit, U.S.A.) were added just before the start of incubation. Preparation to be tested for immunosuppresive activity were added in 0.01 ml at time of several conditions. Cells were incubated for 48 hr at 37°C in a humidified 5% $CO_2$ atmosphere, after which time 0.5 $\mu$Ci of $^3$H thymidine (Radiochemical Centre, Amersham. England, specific activity 5 Ci/mmol) was added to the cultures. 24 hr later cultures were collected on glass fiber filters using automatic multiple sample harvester. Label incorporation was measured by liquid scintillation counting in a Beckman spectrometer.

Controls for the lymphocyte experiment consisted of cells in the presence and absence of PHA, and cells with the PHA and control protein (usually human albumin.)

### *Isolation of human AFP*

Human AFP was purified from the serum of patient with hepatoma by affinity chromatography, according to the method of Nishi[13].

The r-globulin fractions of rabbit antisera were coupled to cyanogen bromide-activated Sepharose-4 B at pH 5.9 in 0.2 M citrate buffer with 0.5 M NaCl and columns were equilibrated in PBS with 0.15 M NaCl. Bound proteins were eluted in 0.2 M glycine-HCl buffer, pH 2.8 with 0.5 M NaCl; immediately neutralized; and dialyzed exhaustively against PBS. Minor contaminants were removed via a Sepharose anti-normal adult serum column.

Samples were concentrated in an Amicon-positive pressure system.

The criteria of purity was a single band on sodium dodecyl sulfate polyacrylamide gel electrophoresis and a single precipitin arc on gel diffusion and immunoelectrophoresis using a potent polyvalent antiserum.

### *Iodination of AFP*

AFP was iodinated (with reductant free Na $^{125}$I DAIICH, Japan; specific activity, 197.75 mCi/ml), by the chloramine-T method of Hunter[14] preceeding

each experiment. Iodinated AFP was separated from free iodine by gel filtration on Sephadex G-25. Final protein concentration and specific activity were determined by spectrophotometry and $\gamma$-counting (Packard), respectively. Sufficient unlabeled AFP were added for a final specific activity of 20,000 cpm/$\mu$g of AFP. All reagents and procedures were kept sterile.

*Measurement of binding of $^{125}I$ labelled AFP to lymphocyte*

The bindings of $^{125}I$ labelled AFP to lymphocytes were determined by using a modification of the method of Milton *et al*[15]. One million lymphocyte, suspended in 0.1 ml of Eagle's MEM were incubated in triplicate with increasing concentration (0 to 30 $\mu$g/ml) of PHA-P. 50 $\mu$g of $^{125}I$ labelled AFP were added simultaneously in the each culture. After incubation for 30 min. at 37°C, the reaction was terminated by adding 3 ml of cold phosphate buffer saline. Following removal of unbound $^{125}I$ labelled AFP by washing four times with Eagle's MEM at 0°C. Cell were extracted, collected on Millipore filters (HAWP 304 $\phi$ 25 mm), and the radioactivity was measurred in auto gamma counter. Nonspecific binding was assessed from blanks containing no cells.

*Characterization of $^{125}I$ labelled AFP-PHA complex*

Complexes were formed using a excess of PHA relative to that required for optimal stimulation, (i. e. 200 $\mu$g of PHA-P. with 100 $\mu$g of $^{125}I$ labelled AFP in 1 ml of phosphate buffer saline). After 1 hr at 37°C, 0.2 ml of mixture were applied on a column Sephadex G-200 (1.5 × 100 cm) previously equlibrated with 0.01 M Tris-HCl buffer, pH 8.0. Fractions of 1 ml were collected and radioactivity was measured in an auto gamma counter.

## RESULTS

*Effect of AFP on PHA-response of normal lymphocytes*

The dose-dependent suppression of PHA stimulation of lymphocytes for AFP is shown in Figure 1. The results demonstrated that AFP strongly inhibited the PHA response of normal lymphocytes at a concentration of 200 $\mu$g/ml and produced linear dose response curve between 10 $\mu$g/ml and 200 $\mu$g/ml. None of the suppressive effect has observed by addition of human albumin at concentrations of 2 mg/ml as a control (not shown). In general, therefore, the effects of our preparation of AFP on PHA induced lymphocyte transformation are similar to the results of other investigators.

*Preincubation of lymphocytes with AFP before stimulation*

In order to examine the persistence of the supperssive activity, lymphocytes were preincubated with AFP at a concentration of 100 $\mu$g/ml for

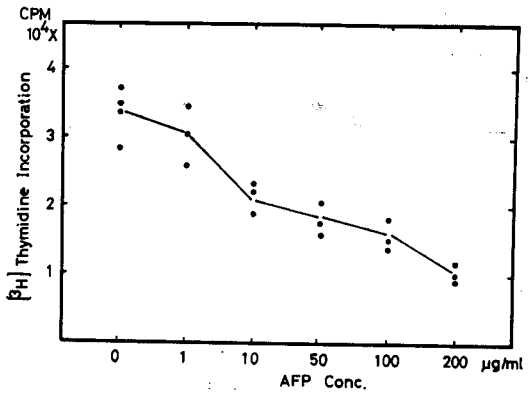


**Fig. 1.** Dose response effect of purified AFP on PHA-induced lymphocyte transformation.

**Table 1.** *Effect of preincubating lymphocytes with AFP on PHA-induced lymphocyte transformation*

| Cultures | Mean up take ± S.D. | Control (%) |
|---|---|---|
| Preincubation with AFP | | |
| washing (−) | 26,321 ± 1,838 | 64.9 ± 4.5 |
| washing (+) | 30,208 ± 1,974 | 74.6 ± 4.9 |
| Preincubation with albumin | 40,650 ± 601 | 100.3 ± 1.5 |
| Preincubation negative | | |
| AFP (+) | 24,295 ± 2,136 | 60.0 ± 5.3 |
| AFP (−) | 40,949 ± 3,560 | |

2 hr. After removal of the AFP, containing culture medium and washing (three times), the cell were stimulated by PHA. Control cultures were pre-incubated for similar lengths of time with albumin.

The response of lymphocytes to PHA after preincubation with AFP is seen in Table 1. After a 2 hr exposure to a suppressive concentration of AFP and its removal by washing, lymphocytes responded suppressively. There was residual effect of AFP upon the ability of lymphocytes to respond to PHA. The suppressive effect of AFP could be reduced slightly by washing the lymphocyte after incubation period.

*Time course of suppressive effect by AFP*

To determine if the AFP interfered with some aspect of cell metabolism which occurs subsequent to activation, attempts were made to inhibit lymphocyte proliferation with AFP after the stimulatory response had begun. AFP was added to lymphocyte cultures in 100 μg/ml concentration at various

**Table 2.** *Effect of delayed addition of AFP on PHA-induced lymphocyte transformation*

| Hours AFP added after PHA | Mean uptake ± S.D. | Control (%) |
|---|---|---|
| 0 hr. | 24,295 ± 2,136 | 60.0 ± 5.3 |
| 1 hr. | 27,536 ± 2,422 | 68.0 ± 5.9 |
| 3 hr. | 29,623 ± 2,156 | 73.2 ± 5.3 |
| 24 hr. | 38,469 ± 3,021 | 94.9 ± 7.4 |
| Control culture | | |
| AFP (+) | 24,295 ± 2,136 | 60.0 ± 5.3 |
| AFP (−) | 40,494 ± 3,562 | |

times after PHA was added. The results are presented in Table 2, and show that with respect to the intervals chosen, maximal inhibition of $^{3}H$-thymidine uptake occurred when the AFP was added at same time with PHA. There were still inhibitory effects when AFP was added as late as 3 hr after PHA stimulation. The possibility that AFP may prevent the attachment of PHA to lymphocytes and thus inhibit the PHA response was studied by preincubating the lymphocyte with PHA for 1 hr to allow PHA binding before the addition of AFP to the culture. Under these conditions, the degree of inhibition of PHA response was almost the same as that shown when AFP was added to the culture with PHA simultaneously.

The lymphocytes which had AFP added after 24 hr exposure to PHA proliferated at a rate similar to those lymphocytes which had no AFP. Thus the AFP did not prevent the proliferation response of the lymphocytes once they had been "activated" by PHA.

*Effect of PHA on $^{125}I$ labelled AFP binding to lymphocyte*

On the basis of the studies reported above, AFP appeared to involve the early events associated with stimulation. To investigate the mode of action of AFP on the lymphocyte surface, the binding studies of $^{125}I$ labelled AFP were performed on its interaction with the different concentration of PHA.

The pattern of AFP binding curve is shown in Figure 2. The detectable $^{125}I$ labelled AFP molecules bind to the lymphocyte surface in the absence of PHA and progressive binding of $^{125}I$ labelled AFP occurred with increasing concentration of PHA. Equilibrium binding plateaus were reached by 30 $\mu$g/ml of PHA and remained for at least 50 $\mu$g/ml. No competition of $^{125}I$ labelled AFP binding occurred with increasing excess of PHA on the PHA receptors of lymphocyte surface. AFP simply binds to the lymphocyte

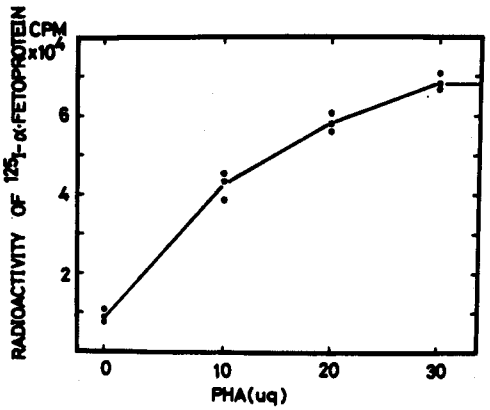


**Fig. 2.** Attachment of $^{125}$I-AFP to lymphocyte with PHA.

surface itself. Moreover, the presence of PHA promotes the binding of AFP.

*Fractionation of $^{125}$I labelled AFP-PHA complex*

PHA was incubated with $^{125}$I labelled AFP under the normal conditions of culture except that 10 fold larger quantities were used in order to have sufficient material to study the molecular nature of the product. Figure 3 shows the elution profile of mixed materials from a column of Sephadex G-200 calibrated with standared proteins. PHA incubated with $^{125}$I labelled AFP gave one rather peak whose position corresponded to a molecular weight of about 200,000 daltons, whereas either PHA and AFP alone eluted as a lower molecular weight component. As a result, it appears that AFP (mol. wt. 70,000 daltons) and PHA (mol. wt. 135,000 daltons) make the complex.

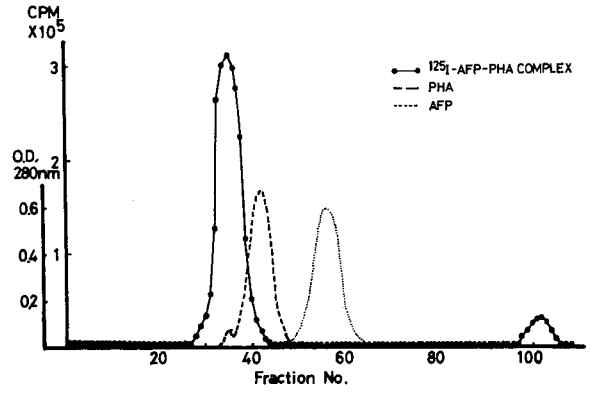


**Fig. 3.** Gel filtration pattern of $^{125}$I-AFP-PHA complex on sepharose 6-B.

## DISCUSSION

The major purpose of this study was to investigate the mode of action of non-specific immunosuppression by human AFP. Before discussing the results which directly focus on this problem, two observations which may explain why suppression is not found uniformly with every preparation of AFP bear mentioning. Based on our experiments that it is difficult to remove albumin from AFP fraction by the usual method using affinity chromatography. In addition, the modification such as disialylation, of AFP molecule may occur during the purification steps. These could potentially introduce serious errors into the experiments of immunosuppression of AFP.

Several investigators have been reported that AFP suppresses lymphocyte proliferation in vitro, after stimulation by mitogenesis[3~6,9]. In this investigation, we first demonstrated the non-specific immunosuppressive activity of human AFP. Highly pure and native AFP effectively inhibited normal lymphocyte transformation by PHA. Dose-response studies demonstrated that AFP was still suppressive at a concentration of 10 $\mu$g/ml, and produced linear dose-response curve between 10 $\mu$g/ml and 200 $\mu$g/ml (Fig. 1).

The concept that AFP may have an important immunoregulatory role is very attractive because it offers the explanation for the survival of the mammalian fetus in a histoincompatible environment and the immunological hyporeactivity of pathological conditions. However, little work has been done on its mechanism by which AFP blocks the lymphocyte transformation with non-specific or specific antigens in vitro. Therefore, we extended this concept by demonstrating the mode of action of non-specific immunosuppression by human AFP.

Exposure of the cells to suppressive concentrations of the AFP for 2 hr before stimulation did not appear to alter appreciably their responsiveness. In addition, the AFP did not appear to alter the cellular response which occur after lymphocyte have undergone "activation". We conclude from these results that AFP acts at about the time that "activation" of lymphocyte occurs. (Table 1, 2)

It has been suggested by a number of authors that "activation" of lymphocyte is an essential part of the cellular immune system and is a consequence of reactions to antigen recognition. It has been hypothesized that the activation phenomena are mediated by receptor sites on the plasma membrane of lymphocyte. On the other hand, the action of human immunoregulatory $\alpha$-globulin (IRA) on lymphocyte cultures, described by Cooperband *et al*,[10] had the characteristics in which small amounts of IRA could be bound weakly to the lymphocyte surface, but were easily removed by wash-

ing. Further, Lineweaver-Burke analysis indicated that PHA and IRA reacted with different receptors on the cell surface.

Since AFP is not easily removed by washing, this interaction of AFP with lymphocyte suggests one of moderate affinities. In addition competitive analysis between PHA and radiolabeled AFP indicates that there is non competitive interaction between these agents. Moreover, progressive binding of radiolabeled AFP occurred with increasing concentration of PHA (Fig. 2). Therefore, AFP may inhibit PHA mitogenesis by binding with the some region of the lymphocyte surface glycopeptidyl oligosaccharide "receptors" as the PHA combining site. AFP whose carbohydrate moieties are not well characterized will serve as a source of glycopeptides that can be used to determine the structure of the site on the lymphocyte surface where PHA induces mitogenesis.

Under steady state conditions, however, AFP-PHA complexes were observed by gel filtration columns (Fig. 3). Such complexes suggest that AFP has some specific affinity for the PHA because of its carbohydrate moieties. It is concluded that the complex of AFP, PHA, and PHA receptor, interfere with the lymphocyte activation.

Some glycoproteins also inhibit mitogenic responses of lymphocytes induced by PHA. The hapten inhibitors can remove PHA from the lymphocyte cell membrane after it has bound to the surface receptor sites. In fact, Mendelsohn[17] had reported that the glycoprotein fetuin can rapidly release bound PHA from lymphocytes even after the PHA had been in contact with the cells for 3 hr. The mode of action by which AFP interefere lymphocyte transformation is completely different as mechanism of hapten inhibitors.

AFP may be associated with immunosuppressive activity. However, this may not be an intrinsic characteristic of the protein itself, but rather a reflection of its ability to combine with antigens and lymphocyte surface. In vivo observation are limited to date, but the evidence that AFP may serve as an important immunoregulatory protein is not conclusive. Further studies are necessary to define the in vivo mechanism(s) involved in AFP induced suppression.

## COCLUSION

The ability of alpha-fetoprotein (AFP) of mouse or human origin to suppress certain T cell dependent immune reactions is well documented. However, the mechanism by which AFP suppresses the immune reaction has not been defined.

In the present study the inhibitory activity and the mode of action of

AFP on PHA-induced normal lymphocyte transformation was investigated and the following observations were made. (1) AFP inhibited the PHA response of normal lymphocyte at a concentration of 100 $\mu$g/ml and produced a linear does response curve between 10 $\mu$g/ml and 200 $\mu$g/ml. (2) The response of lymphocyte to PHA after preincubation with AFP was still suppressive. (3) AFP was added to the culture of PHA stimulated lymphocyte at sequential incubation time and maximal inhibition of $^{3}$H-thymidine uptake occurred when AFP was added simultaneously to PHA stimulation. (4) Addition of AFP did not interfered the binding of PHA to the lymphocyte surface but rather increased the amount of PHA molecules bound. (5) AFP-PHA complex was observed on a column gel filtration. Thus, the complex of AFP, PHA and PHA receptor interferes the lymphocyte activation.

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